DNA GLOBALFILER AMPLIFICATION SETUP USING THE EPMOTION

A. SCOPE

A.1 The GlobalFiler kit is a multiplex short tandem repeat (STR) system for use in DNA typing. This kit has a 6-dye configuration and amplifies 21 autosomal STR loci, Amelogenin, 1 Y-STR locus (DYS391), and 1 Y insertion/deletion (Y-indel) locus. The 21 autosomal STR loci are D3S1358, vWA, D16S539, CSF1PO and TPOX (labeled with 6-FAM); D8S1179, D21S11 and D18S51 (labeled with VIC); D2S441, D19S433, TH01 and FGA (labeled with NED); D22S1045, D5S818, D13S317, D8S820 and SE33 (labeled with TAZ); and D10S1248, D1S1656, D12S391 and D2S1338 (labeled with SID). The Amelogenin, DYS391 and the Y-indel markers are labeled with the VIC fluorescent dye. Ten of these STR loci are classified as mini-STRs because they are less than 220 bp and as such can perform well with degraded samples. All 24 loci are amplified simultaneously in a single tube and can be analyzed in a single injection on an AB 3130 capillary electrophoresis

A.2 The amplification setup process consists of multiple transfers of liquids containing either reagents or DNA from one place to another. By utilizing the epMotion 5075, a liquid handling robot, the incidence of human error and/or the introduction of contamination in this process can be minimized. Furthermore, automation of the amplification setup process allows for analysts to complete other tasks while these steps are being performed.

B. QUALITY CONTROL

B.1 Positive amplification/allelic control (e.g. 007, 2800M etc.)

This sample ensures that the amplification and typing process is working properly. It is required to run a positive amplification control with each GlobalFiler amplification. For the epMotion, the 007 sample must be transferred out of the manufacturer's tube into a QIAcube elution tube or regular manual extraction tube (these manual tubes have holes in their snap caps), the 2800M does not need to be transferred.

B.2 Negative controls:

Reagent Control: This is a tube containing no sample that is carried through the DNA typing process, involving all the reagents used for extraction, quantitation, and amplification. The purpose of this sample is to detect contamination that might occur from the reagents, the environment, or between the evidence samples being processed. At least two reagent controls will be extracted per extraction set, except during the extraction of reference samples where one reagent control may be extracted. All reagent controls will be quantitated, with the reagent control demonstrating the greatest signal being amplified and typed. A reagent control that is amplified and typed shall be amplified utilizing the same primers, instrument model, and concentration conditions as required by the evidence sample with the least amount of DNA; amplified with each amplification kit utilized; and typed using the same instrument model and injection conditions (i.e. injection times and voltage) as the associated evidentiary sample containing the least amount of DNA.

If an evidence sample is re-amplified with the same amplification test kit or system and the template volume is not increased over that of the original reagent control, then re-amplification of the associated reagent blank is not necessary.

Negative amplification control: This control contains only the reagents used to prepare the PCR amplification mixture for each batch of samples, including sample buffer (TE^{-4}). The purpose of this sample is to detect contamination that might occur from the PCR reagents, the PCR setup environment or between the PCR reactions being prepared. It is required to run a negative amplification control with each amplification.

B.3 See DOC ID 12626 regarding processing water controls.

B.4 A lab coat, mask, and protective gloves must be worn during amplification setup to prevent contamination.

B.5 Decontaminate the bench work area with a bleach-based cleaner, e.g. Clorox Bleach Germicidal Cleaner, before and after amplification setup.

B.6 Decontaminate the epMotion deck with Decon Quat cleaning solution followed by 70% ethanol, before and after amplification setup.

B.7 Decontaminate the Eppendorf 24TC racks with 70% ethanol after amplification setup.

B.8 Decontaminate the waste container with a bleach-based cleaner, e.g. Clorox Bleach Germicidal Cleaner, after amplification setup.

B.9 See DOC ID 1835 to determine reagent expiration dates.

B.10 Each new lot of GlobalFiler must undergo quality control testing prior to being used for the amplification of casework samples; see DOC ID 12626 for more information about this testing.

B.11 Each new lot of TE^{-4} must undergo quality control testing prior to being used to dilute casework samples; see DOC ID 12626 for more information about this testing.

B.12 Amplification setup must be performed in the pre-amplification room. Amplification plate bases should not be brought into the post amplification room.

C. SAFETY

C.1 Protective gloves, a lab coat, and a mask must be worn during plate setup. Additionally, eye protection (e.g. safety glasses or a face shield) must be worn when working with any reagents or DNA extracts outside of the epMotion.

C.2 All appropriate SDS sheets must be read prior to performing this procedure.

C.3 Treat all biological specimens as potentially infectious.

C.4 Distinguish all waste as general, biohazard, or sharps and discard appropriately.

D. REAGENTS, STANDARDS AND CONTROLS

D.1 GlobalFiler

D.1.1 Pre-amplification

D.1.1.1 GlobalFiler Master Mix

D.1.1.2 GlobalFiler Primer Pair Set

D.1.1.3 DNA Control 007 (0.1 ng/µL) or 2800M Positive Control (10 ng/µL or 0.1 ng/µL)

D.1.2 Post-amplification

D.1.2.1 GlobalFiler Allelic Ladder Mix

D.1.2.2 GeneScan LIZ 600 Size Standard v2.0

D.2 Clorox Bleach Germicidal Cleaner (Decontamination)

D.3 Decon Quat (Decontamination)

D.4 70% Ethanol (Decontamination)

D.5 TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, 1L)

Add 10 mL 1 M Tris-HCl, pH 8 and 150 µL 0.5 M EDTA to 990 mL deionized water. Store at room temperature.

E. EQUIPMENT & SUPPLIES

E.1 Equipment

E.1.1 epMotion 5075 (instrument, computer, and appropriate software)

E.1.2 epMotion dispensing tools

E.1.3 epMotion labware (thermoblocks, 24TC racks, module racks, and reservoir rack)

E.1.4 Thermal cycler

E.1.5 Microcentrifuge

E.1.6 Pipettes

E.1.7 Vortexer

E.1.8 96 well-plate centrifuge

E.2 Supplies

E.2.1 epMotion supplies (30 mL reservoirs, epT.I.P.S. Motion 1-50 µL tips, epT.I.P.S. Motion 20-300 µL tips, and epT.I.P.S. Motion 40-1000 µL tips)

E.2.2 Sterile aerosol-resistant tips

E.2.3 Microcentrifuge tubes racks

E.2.4 AB optical 96-well plates

E.2.5 AB plastic strip caps

E.2.6 96-well plate base

E.2.7 Disposable gloves

E.2.8 Mask

E.2.9 Lab coat

E.2.10 Eye protection (e.g. safety glasses, face shield)

E.2.11 Permanent marker

DNA Analysis Workbook (optional)

F. PROCEDURES

F.1 Ensure that the standard curve utilized to determine sample concentration meets the requirements in DOC ID 1785 prior to determining the volume of DNA to amplify. When possible target approximately 0.25 -0.75 ng. Amounts greater than 0.75 ng may also be utilized if for example, a mixture is indicated, a sample is degraded, etc. Fill out an amplification sheet. If 50% or less of a sample has been consumed and the quantity of DNA is less than 0.25 ng, amplification will not be performed; instead, a request to consume the sample will be made or additional sample, up to 50%, will be taken. An exception to this can occur if the quantity obtained is very close to the lower end of the target range, e.g. 0.24 ng. In addition, if a sample has been consumed and the total quantity of DNA that can be input into a reaction is less than 0.010 ng, amplification with GlobalFiler does not have to be performed

F.2 Normalization calculations should be done prior to setting up the epMotion worktable.

F.2.1 Copy and paste the Plexor HY quantitation data (sample names and corresponding autosomal concentrations) into columns D and E of the Excel worksheet named "GlobalFiler.xlsm". Also, enter the amplification positive control(s) with the appropriate concentration (i.e. 0.1 ng/µL or 10 ng/µL) and the negative amplification control (TE^{-4}) with a 0 ng/µL concentration.

Note: *If two positive controls are being run, these must be in two separate tubes and placed in the thermorack following the placement of the sample tubes. A minimum volume of 10.0 µL of the 007 positive control sample must be transferred out of the manufacturer's tube into QIAcube elution tubes or regular manual extraction tubes (these manual tubes have holes in their snap caps) before placement. The 2800M positive control does not need to be transferred into a different tube; because of its 10 ng/µL concentration it will undergo a 1:100 dilution prior to addition into the final PCR plate.*

The negative control containing a 20 µL aliquot of the TE^{-4} being used for dilution should also be in a separate tube and placed in position after the positive controls.

F.2.2 Add the appropriate amount of DNA (typically 0.25 – 0.75 ng) into column F of the worksheet.

F.2.3 Click on the purple "Convert N/A" button; this will run a macro that will convert N/A to 0 so that the maximum volume of sample will be added to the final PCR plate.

F.2.4 The worksheet will highlight in yellow any samples that do not have enough DNA to meet the target amount and highlight in pink any samples that need to be diluted 1:10 or 1:100.

Note: *There are formulas in place such that if the required volume ("Vol Ampl") is less than 1 µL, then a dilution will take place. If this volume is less than 0.1, then a 1:100 dilution will be triggered ("Dilution Ratio"); if the volume is between 0.1 and 1, then a 1:10 dilution will be triggered. Otherwise, the cell will remain blank if the volume is larger than 1 µL.*

F.2.5 The worksheet will outline in red any dilution samples that require a volume of less than 1 µL to be added to the final PCR plate; these samples may be diluted manually offline. For example, a sample with a concentration of 750 ng/µL would require 0.1 µL of a 1:100 dilution to be added to the final plate to target 0.75 ng; however, by diluting this sample 1:10 offline (to a concentration of 75 ng/µL), an allowed volume of 1.0 µL could instead be pipetted by the epMotion onto the final PCR plate.

F.2.6 The worksheet will also highlight in orange any samples that require a volume of less than 1 µL of TE^{-4} for PCR.

F.2.7 Once the Normalization List tab has been completed, three CSV files must be created to import into the epMotion amplification setup method.

F.2.7.1 Click on the **"Save TE as CSV"** button and then save the CSV file with an appropriate name including "TE". Click OK when a message regarding saving 'Book 1' with references to unsaved documents appears. This macro is designed to remove all empty rows based on the highlighted selection and save as a CSV. This single CSV file will add TE^{-4} to the final PCR plate for PCR normalization and the dilution plate for dilutions (either 27 µL or 198 µL).

F.2.7.2 Click on the **"Save Sample as CSV"** button and save the CSV file with an appropriate name including "Sample". This macro is designed to remove all empty rows based on the highlighted selection and save as a CSV. This single CSV file will add samples to all necessary locations on the final PCR plate for PCR normalization and the dilution plate for dilutions (either 2 µL or 3 µL).

F.2.7.3 Click on the **"Save Dilutions as CSV"** button and save the CSV file with an appropriate name including "Dilutions". This macro is designed to remove all empty rows based on the highlighted selection and save as a CSV. This single CSV file will add the diluted samples to the final PCR plate.

F.2.7.4 Save the entire Excel workbook with an appropriate name including your initials and the date. Click Yes when a message regarding macro-free workbooks appears.

F.2.7.5 After saving the workbook, modifications can be made to its appearance (e.g. column width) by unprotecting it. This unprotected workbook can be saved with a different name than the macro-enabled workbook. In addition, any samples that have been or need to be diluted offline (see F.6.5) and had their concentration changed accordingly in the workbook, should be highlighted in blue.

F.3 Determine the number of reactions to be setup including two amplification positive controls and one amplification negative control. Add additional reactions to this number to compensate for loss during pipetting (six extra samples are needed for a full epMotion amplification plate, i.e. 88 sample extracts). See the below chart for the volume of each component needed per reaction.

Component	Volume (Per Reaction)
TE^{-4}	To a final volume of 25.0 µL
GlobalFiler Master Mix	7.5 µL
GlobalFiler Primer Pair Mix	2.5 µL
Template DNA (0.25-0.75 ng)	Up to 15.0 µL
Total reaction volume	**25.0 µL**

F.4 Open the Eppendorf eBlue software.

F.5 Select **Application Editor** from the main menu, from the **DNA** user tab choose the **amplification** folder and then select **GlobalFiler-small** or **GlobalFiler-large** method in the Applications list.

F.5.1 To reduce evaporation of sample extracts and GlobalFiler reagents during the setup of a large amplification plate, the **GlobalFiler-large** method should be utilized. Unlike the **GlobalFiler-small** method, this method has a User Intervention step after the last TE^{-4} transfer and prior to the first sample extract transfer. At this step the method will stop and the user will be prompted to cap the GlobalFiler reagents and uncap all sample extracts and amplification positive and negative controls before proceeding.

F.6 The **GlobalFiler-small** and **GlobalFiler-large** methods must be modified with every amplification plate performed.

F.6.1 Since these methods are "read only", select **File - Save As Icon** and name the application with your amplification run name so that the following method modifications can be made.

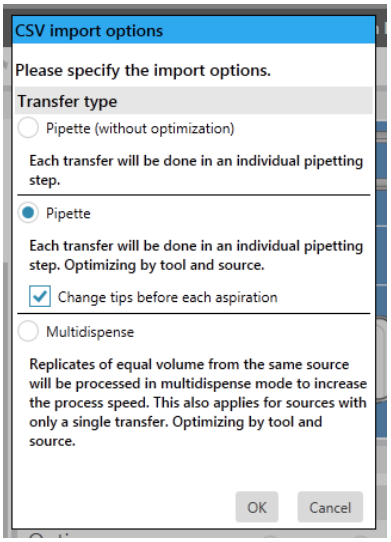
F.6.2 Click on the **Switch to Procedure** Button and then click on **Step 2 - Reagent Transfer**. Using the reagent calculations from F.3, enter the volume of GlobalFiler Master Mix to pipette in the Volume location. If this number is $\leq$ 300 µL change the **Pipet Tool** in this reagent transfer step from the TS_1000 to the TS_300. Click the **Save Icon**.

F.6.3 Click on **Step 4 - Reagent Transfer**. Enter the volume of GlobalFiler Primer Pair Set to pipette in the **Volume** location. If this number is $\leq$ 50 µL change the **Pipet Tool** in this reagent transfer step from the TS_300 to the TS_50. Click the **Save Icon.**

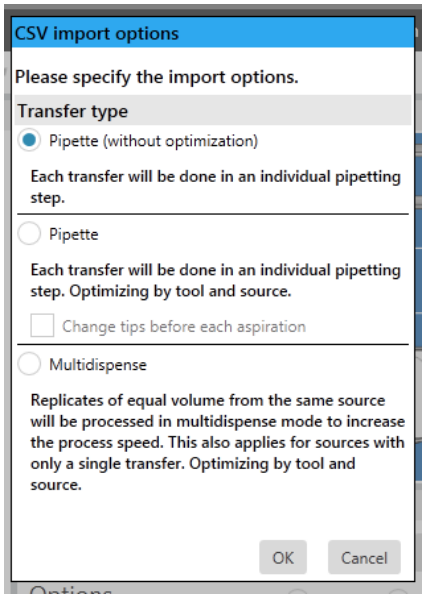
F.6.4 Click on **Step 5 - Number of samples**. Enter the number of wells that the GlobalFiler reaction mix should be transferred to on the final PCR plate. Click the **Save Icon**

F.6.5 To import the three required CSV files follow the three comment icons listed in the procedure. Click on the step immediately following each comment icon highlighting that step in blue. Then click the csv icon on the tool bar to import each file starting with the TE^{-4} CSV file.

F.6.6 After each CSV file is uploaded the following pop up will prompt the user to choose the pipetting options. For TE^{-4} and dilutions CSV select as follows:

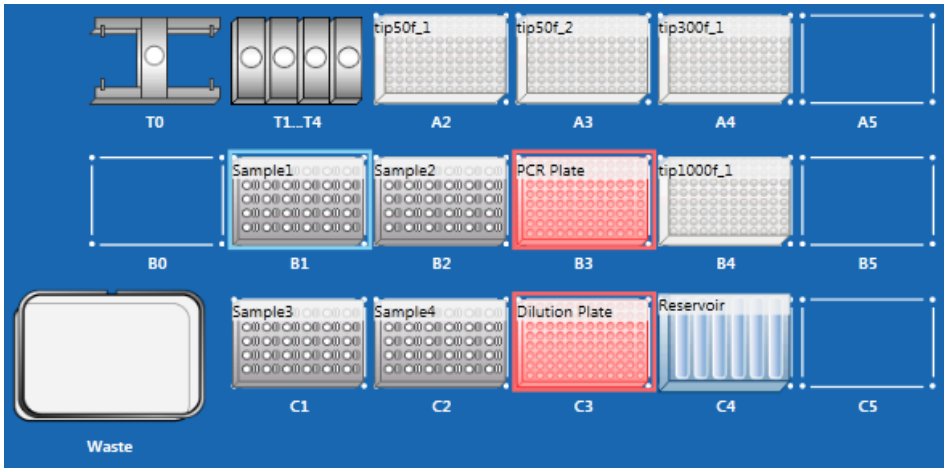


For samples CSV select as follows:



F.6.7 On the last TE^{-4} transfer step (before the first sample extract transfer step) select the **Options** tab and under **Change Tips** select "...before asp. for next destination, well…"; otherwise, a tip with a small amount of TE^{-4} will be used for the first sample extract transfer. Click the **Save Icon**.

F.7 Prepare the epMotion worktable using the following diagram and instructions for guidance:



F.7.1 Place epT.I.P.S. Motion 1-50 µL tips in positions A2 and A3 (A2 and A3 are denoted as "tips50f_1" and "tips50f_2", respectively), epT.I.P.S. Motion 20-300 µL tips in position A4 (A4 is denoted as "tips300f_1"), and epT.I.P.S. Motion 40-1000 µL tips in position B4 (B4 is denoted as "tips1000f_1") as shown above. The robot can use two types of tip versions. If using the older version the operator **MUST VERIFY <u>that the box has sufficient tips for pipetting and has no tips out of place from the first tip position.</u>**

F.7.2 Ensure that the epMotion dispensing tools needed for this protocol, i.e. the TS 50 single channel, TS 300 single channel, and TS 1000 single channel tools, are in place on the worktable; these tools can be placed in any sequence on the tool holders in position T1…T4.

F.7.3 Place Eppendorf 24TC racks in positions B1, B2, C1, and C2 on the worktable as needed; these are denoted as "Sample1", "Sample2", "Sample3", and "Sample4", respectively. Alternatively, these racks can be added with the sample extract tubes after the user intervention step when the **GlobalFiler-large** method is utilized; keeping the racks refrigerated will minimize evaporation.

F.7.4 Place thermoblocks in positions B3 and C3 (denoted as "PCR Plate" and "Dilution Plate") and the reservoir rack in position C4 (denoted as "Reservoir").

F.7.5 Place empty 96-well plates on these two thermoblocks.

F.7.6 Prepare the reagent reservoir rack:

F.7.6.1 Ensure that a 1.5/2 mL module rack is located in both the third and seventh position of the reservoir rack as shown below.

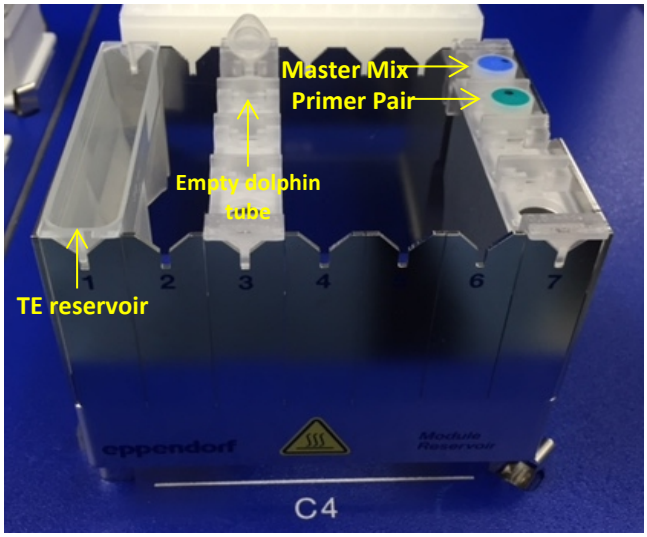
F.7.6.2 Place an open 30 mL reservoir with TE^{-4} in the first position of the reservoir rack.

F.7.6.2.1 10 mL of TE^{-4} will be more than enough volume for a typical amplification plate; this would cover 40 dilutions (1:100) and the normalization of 96 samples. If 90 samples needed a 1:100 dilution, approximately 20 mL of TE^{-4} would be needed for both dilution and normalization.

F.7.6.3 Place an open empty dolphin tube in position 3A of the reservoir rack.

F.7.6.4 Mix the GlobalFiler reagents by vortexing each tube for at least 3 seconds before each use; these tubes may be centrifuged briefly before opening. Ensure that the volume in each of the reagent tubes is sufficient for the volumes calculated in step F.3.

F.7.6.4.1 Place the closed GlobalFiler Master Mix tube in 7A and closed GlobalFiler Primer Pair Set tube in 7B of the reservoir rack; see photo below.



F.8 Place open sample extract tubes in racks B1 (Samples 1-24), B2 (Samples 25-48), C1 (Samples 49-72), and C2 (Samples 73-96), as necessary if using the **GlobalFiler-small** method. Sample extract evaporation can be limited by storing these racks in the refrigerator prior to use. If using the **GlobalFiler-large** method, closed sample extract tubes can be placed in the appropriate racks or these open sample extract tubes can be added after the user intervention step.

Note 1: *Sample extracts must be in QIAcube elution tubes or regular manual extraction tubes (these manual tubes have holes in their snap caps). Dolphin tubes fit very tightly in the Eppendorf 24TC rack and therefore, often get stuck; if a sample has been concentrated into a dolphin tube prior to quantitation, transfer this sample extract into one of the aforementioned suitable tubes prior to using the epMotion.*

Note 2: *The closed sample extract tubes can be placed on the epMotion deck at any point prior to step F.8; however, the tubes should not be opened until this step (F.8) to avoid evaporation.*

F.8.1 The racks are numbered for sample order; sample extracts 1-6 should be placed in the first row, sample extracts 7-12 in the second row, and so on.

***Note**: The Sample Layout tab in the worksheet is a helpful guide that shows where sample tubes should be placed on the thermoracks in a row-wise fashion according to the Normalization List.*

The PCR Plate Layout tab is a helpful guide that shows the layout of the reactions on the final PCR plate as well as where the dilutions will occur on the dilution plate.

F.9 Place an open 007 positive control tube(s) (that has been transferred out of the original manufacturer's tube) or an open 2800M positive control tube(s) and an open negative control tube (containing TE^{-4}) after the sample extracts in the appropriate positions of the appropriate Eppendorf 24TC rack.

F.10 Open all tubes containing amplification reagents on the epMotion worktable.

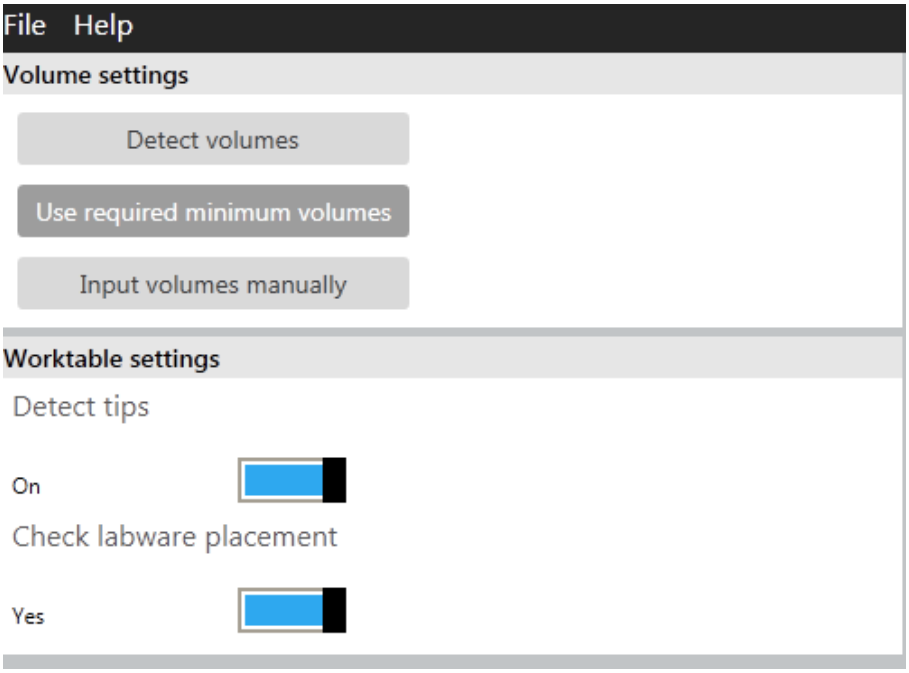
F.11 Close the front hood of the epMotion.

F.12 An epMotion deck check (which includes sample extract order verification) should be completed by a second DNA analyst whenever possible, i.e. during normal business hours when another qualified analyst is available.

F.13 Click the **Run Icon**

F.14 Ensure that compatible devices is selected and that device 5075ZN301615 is highlighted, click **Next.**

F.15 Ensure that **Use required minimum volumes, Detect tips, and Check labware placement** are selected on the following screen



***Note**: The level sensor will check the type and quantity of tips present on the worktable. However, this sensor does not determine if there are enough tips for the entire run; the method will continue until all tips have been used and then the process will stop and the software will prompt you to insert more tips.*

F.16 Click **Run, and the application will begin**

***Note 1**: The expectation is that no significant volume of sample extract will remain in a tube containing 16.0 µL after a 15.0 µL transfer due to loss in pipetting and/or extract evaporation during setup; therefore, these samples may be treated as having been consumed.*

F.16.1 To stop the method before it is complete, click the **Pause** and or **Stop** icon (red square) in the Control tab or lift the front hood. Then click the **Abort** icon (red circle with an "x") to abort the method; the front hood must be down to abort the method. Alternatively, after stopping the method, click the **Resume** icon

F.17 After completion of the method, click **OK** and select file **Exit to Home Screen**, click on **Log Viewer** select your log file by finding the file with the appropriate date and time. Select the **Print Icon** and click PDF to save the file as a PDF document. This file should be saved in the appropriate analyst's casework folder on the I drive. A run completed without errors will have "Program ended successfully" on the last line of the log file.

F.18 Open the front hood and remove the 96-well PCR plate and place it into a plate base; cover wells with plastic strip caps. The wells of the 96-well dilution plate can also be covered with plastic strip caps and this plate retained.

F.19 Cap the GlobalFiler Master Mix and GlobalFiler Primer Pair Mix tubes on the epMotion deck. Place the amplification plate into a thermal cycler and start the GlobalFiler method.

DNA thermal cycling conditions:

Initial incubation:	95 °C for 1 minute
Cycle (29 cycles):	94 °C for 10 seconds (Denature)
	59 °C for 90 seconds (Anneal/Extend)
Final extension:	60 °C for 10 minutes
Final hold:	4 °C

F.20 After the amplification is complete, remove the plate from the instrument block and store the amplified products in a refrigerator protected from evaporation, e.g. these plates can be wrapped in parafilm.

***Note:** The amplified products can be removed from the thermal cycler at any time after reaching 25°C. The amplified products can remain on the thermal cycler overnight or over a weekend at 4°C.*

F.21 Prepare the epMotion worktable for the next user:

F.21.1 Remove the GlobalFiler Master Mix and GlobalFiler Primer Pair Set tubes from the epMotion deck.

F.21.2 Remove the sample extract and amplification positive control tubes from the Eppendorf 24TC rack(s).

F.21.3 Discard any unused GlobalFiler reaction mix and TE^{-4}.

F.21.4 Decontaminate any used Eppendorf 24TC racks with 70% ethanol; store these racks and the 96-well thermoblocks in the refrigerator.

F.21.5 Empty the waste container and decontaminate it with a bleach-based cleaner, e.g. Clorox Bleach Germicidal Cleaner.

F.21.6 Decontaminate the epMotion deck with Decon Quat cleaning solution followed by 70% ethanol.

G. INTERPRETATION GUIDELINES

G.1 Not applicable.

H. REFERENCES

H.1 GlobalFiler PCR Amplification Kit – PCR Setup (Quick Reference), 2015, Applied Biosystems by ThermoFisher Scientific, Inc.

H.2 GlobalFiler PCR Amplification Kit User Guide, 2014, Applied Biosystems by Life Technologies; Developmental Validation included on pages 54-135.

H.3 GlobalFiler Validation Binder 5, Verification of GlobalFiler Amplification Setup method.